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Experimental Second Passage of Chronic Wasting Disease ($CWD^{mule\ deer}$) Agent to Cattle

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Summary

To compare clinicopathological findings in first and second passage chronic wasting disease ($CWD^{mule\ deer}$) in cattle, six calves were inoculated intracerebrally with brain tissue derived from a first-passage CWD-affected calf in an earlier experiment. Two uninoculated calves served as controls. The inoculated animals began to lose both appetite and weight 10–12 months later, and five subsequently developed clinical signs of central nervous system (CNS) abnormality. By 16.5 months, all cattle had been subjected to euthanasia because of poor prognosis. None of the animals showed microscopical lesions of spongiform encephalopathy (SE) but PrP^{res} was detected in their CNS tissues by immunohistochemistry (IHC) and rapid Western blot (WB) techniques. Thus, intracerebrally inoculated cattle not only amplified CWD PrP^{res} from mule deer but also developed clinical CNS signs in the absence of SE lesions. This situation has also been shown to occur in cattle inoculated with the scrapie agent. The study confirmed that the diagnostic techniques currently used for diagnosis of bovine spongiform encephalopathy (BSE) in the US would detect CWD in cattle, should it occur naturally. Furthermore, it raised the possibility of distinguishing CWD from BSE in cattle, due to the absence of neuropathological lesions and to a distinctive multifocal distribution of PrP^{res}, as demonstrated by IHC which, in this study, appeared to be more sensitive than the WB technique.

Keywords: cattle; chronic wasting disease (CWD); deer; transmissible spongiform encephalopathy (TSE)

Introduction

Chronic wasting disease (CWD) is a fatal neurodegenerative transmissible spongiform encephalopathy (TSE), which has been identified in captive and free-ranging cervids (Williams and Young, 1992). Affected animals show accumulation of an abnormal form of prion protein (PrP^{res}) in tissues of the central nervous system (CNS) and lymphatic system. Detection of PrP^{res} in these tissues and characteristic histopathological changes in the brain are the basis of currently available diagnostic methods for TSEs (Hamir *et al.*, 2001b).

CWD has been experimentally transmitted by intracerebral inoculation of affected brain from mule deer into a variety of animal species, including a goat (Williams and Young, 1992). Recently, we reported results from a similar experiment in cattle (Hamir *et al.*, 2001a, 2005a). Only 38% of the inoculated animals showed evidence of PrP^{res} amplification, the incubation periods ranging from 2 to 5 years. Clinical signs were minimal, and the typical TSE lesions in brain were absent.

Since cross-species transmission experiments provide valuable information about the biological characteristics of known TSE agents in a given species, we describe here a second passage of CWD in cattle. The primary objective of this study was to confirm and extend clinicopathological observations from the primary transmission experiment.

Materials and Methods

Cattle and Experimental Procedures

Eight 3-month-old Jersey bull calves were obtained and assigned to inoculated ($n=6$) and

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control ($n=2$) groups. Inoculated calves were housed in a Biosafety Level 2 isolation barn at the National Animal Disease Center (NADC), Ames, Iowa. Husbandry of these animals has been described previously (Hamir *et al.*, 2001b). Personnel wore protective clothing while in the isolation facility and showered before leaving.

Material for inoculation was prepared from the brain of a first-passage CWD-affected animal (no. 1768) from a previous experiment in cattle (Hamir *et al.*, 2001a). The brain material was positive for scrapie associated fibrils (SAF) by negative stain electron microscopy and for PrP^{res} by immunohistochemical labelling (IHC) and Western blotting (Hamir *et al.*, 2001b).

Calves were inoculated intracerebrally with 1 ml of a 10% CWD brain inoculum as described by Cutlip *et al.* (1997). The two control calves were not inoculated.

Sample Collection and Tests for PrP

Animals were killed with pentobarbital and subjected to a complete necropsy. Representative samples of lung, liver, kidney, spleen, salivary gland, thyroid gland, reticulum, rumen, omasum, abomasum, intestines (ileum, colon), adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), tonsil, striated muscle (heart, tongue, masseter, diaphragm), eye, sciatic nerve, trigeminal ganglion, pituitary gland and spinal cord (cervical, thoracic, lumbar) were immersion-fixed in 10% neutral buffered formalin. The brain was cut longitudinally, one-half being fixed in formalin and the remainder frozen (-20 °C). The formalin-fixed brain was cut into coronal sections, 2–4 mm wide. A minimum of 5 brain areas per animal were selected for examination by light microscopy. These were of rostral cerebrum (frontal lobe), hippocampus, midbrain (at the level of superior colliculus), cerebellum, and medulla (at the level of obex). Two sections of spinal cord at each location (cervical, thoracic, and lumbar) were selected. The tissues were processed for routine histopathology, embedded in paraffin wax, and sectioned at 5 μm. The sections were stained with haematoxylin and eosin (HE), or examined immunohistochemically for PrP^{res}, as described previously (Hamir *et al.*, 2004b), with a monoclonal antibody, F99/97.6.1 (O'Rourke *et al.*, 2000). This antibody recognizes PrP sequences conserved in most mammalian species in which natural TSEs have been reported. It should be noted that the immunohistochemical (IHC) procedure used in this study, as in previous studies at this laboratory, did not incorporate a proteinase K (or any other proteinase) digestion step; however, for the purpose of simplicity of nomenclature, the term "PrP^{res}" is used here to describe the abnormal prion within tissue sections.

For immunodetection of PrP^{res}, a rapid Western blot (WB) method described previously was used on frozen brain (midbrain and brain stem) tissue (Schaller *et al.*, 1999). The antibody used in this technique, namely 6H4 was obtained from Prionics (Prionics-Check, CH-8952, Schlieren, Switzerland).

Results

Clinical Signs and Microscopical Findings

Between 10 and 12 months after inoculation, cattle gradually lost appetite and started to lose weight. By the time of euthanasia (14.3–16.5 months after inoculation) they had lost at least 20% of bodyweight and became obviously weak. All but one of the inoculated animals (599) showed abnormal neurological signs, commencing 1 to 5 months after the onset of anorexia. The neurological signs, which were intermittent, included circling (usually to the left) in three animals, grinding of teeth in two, head pressing in one, depression and non-responsiveness to external stimuli in two. The cattle often appeared depressed or non-responsive when undisturbed, but reacted to loud noises either by exaggerated posturing or by collapsing to lateral recumbency. Ultimately, all of the animals became recumbent and unable to rise without assistance.

Within 16.5 months of intracerebral inoculation, all inoculated animals were killed to avoid unnecessary deterioration. The two control animals were killed at 10 and 11 days after euthanasia of the last inoculated animal.

Microscopical examination of HE-stained sections of brain and spinal cord (cervical, thoracic and lumbar) failed to reveal lesions of spongiform encephalopathy in any of the experimental animals. A few isolated neurons with single, clear vacuoles of variable size were seen in the red nucleus of four inoculated animals (593, 595, 590 and 589). Also, the central canal in the caudal medulla of animal 593 showed a focal area of protrusion of neuropil with some glial cell infiltrations into the canal (Fig. 1). Neither increased gliosis nor degenerate neurons were seen in central nervous system (CNS) tissues. Significant lesions, apart from a few sarcocysts in striated muscles, were not observed in any of the animals.

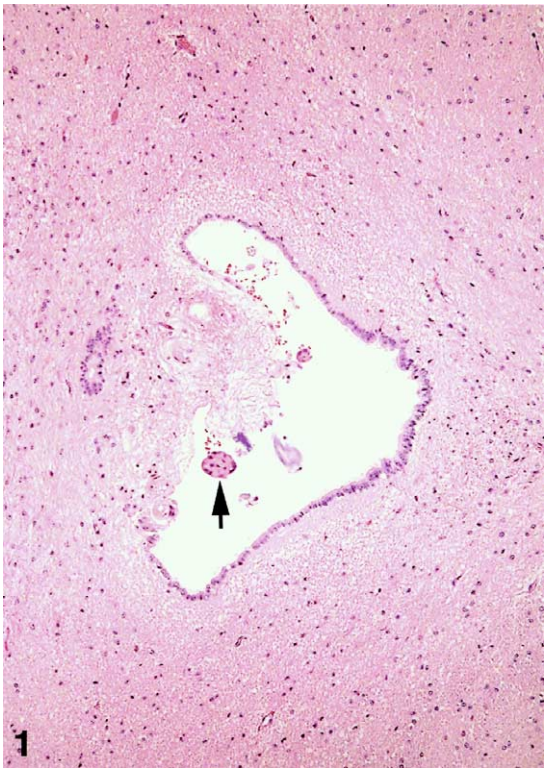

Fig. 1. Brain (caudal medulla) of animal 593. The central canal is partly blocked and distorted. The blockage consists of chronic fibrous inflammation, with a multinucleated giant cell (arrow). HE. ×250.

IHC and WB Findings

The distribution and amount of PrP^{res} IHC labelling within neuronal tissues is summarized in Table 1. There was a consistent pattern of multifocal labelling in brains of all six inoculated cattle (Figs. 2,3). In some tissues, particularly the midbrain, the foci were so numerous and large that they formed large confluent areas (Fig. 3). The labelled foci were located primarily in grey matter but sometimes also appeared in white matter, especially in the rostral cerebrum. Within a focus, the labelled particles occurred individually, in clumps, and occasionally in larger aggregates. They often appeared to be associated with glial cells, either in the cytoplasm or at the cell surface (Fig. 4). The most severely affected tissue was midbrain (Table 1), but the medulla typically showed many labelled foci. Immunoreactivity was less apparent in the hippocampus and rostral cerebrum. Labelling was apparently absent in the cerebellum of four cattle and in the other two inoculated animals was confined to a few small foci in white matter. IHC labelling in spinal cord sections was minimal and, when present, was usually restricted to the cervical cord. No non-CNS tissue, including lymphoid tissues, retina, Gasserian ganglion and pituitary gland showed any immunolabelling (Table 1). Immunoreactivity was absent within neuronal cytoplasm and at perineuronal, and perivascular locations.

WB analysis was performed on samples from the brainstem and midbrain. In brainstem, only three samples (animals 593, 596, 599) were positive by this method whereas samples from the midbrain area of CWD-inoculated animals invariably gave a positive result (Fig. 5), albeit in one animal (590) weak. Samples from the midbrain area of control animals, however, remained negative (Fig. 5). Positive samples showed the typical profile of three bands of proteinase K-resistant isoforms of PrP^{res}, representing the di-glycosylated, mono-glycosylated and unglycosylated polypeptides. Comparison of these results from the second cattle passage with those from the first cattle passage revealed no obvious differences in the molecular weight between the three isoforms of PrP^{res} (data not shown). It should be noted, however, that the unglycosylated isoform of PrP^{res} has a similar staining intensity to that of the mono-glycosylated isoform. This is in contrast to typical bovine spongiform encephalopathy (BSE), in which the unglycosylated isoform shows the weakest staining

Table 1
Immunohistochemical demonstration of PrP^{res} in tissues of second-passage CWD-affected cattle

Ear tag no.	*Immonolabelling of PrP^{res} in*							
	brainstem	*cerebellum*	*midbrain*	*hippocampus*	*cerebrum*	*cervical sc*	*thoracic sc*	*lumbar sc*
590	+	–	++	–	–	+	–	–
593	++	–	++	++	+	–	–	–
599	++	+	+++	++	+	+	+	+
596	+	–	++	+	–	–	–	–
589	++	–	+++	+++	++	+	+	+
595	+	+	+++	+	+	+	+	+
695	–	–	–	–	–	–	–	–
694	–	–	–	–	–	–	–	–

+, Minimal; ++, moderate; +++, extensive. sc, Spinal cord. Retina, trigeminal ganglion and pituitary gland were invariably negative.

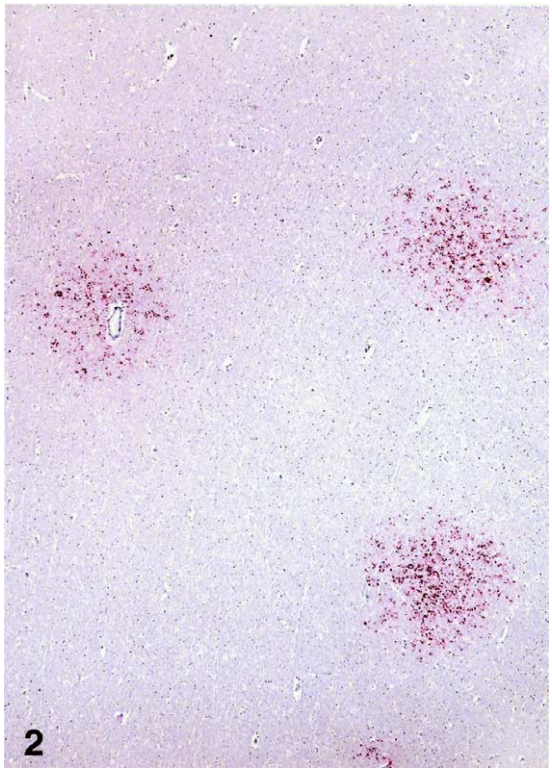

Fig. 2. Brain (rostral cerebral cortex) of animal 589. There are four foci (three large and one small) of PrP^{res}. IHC labelling. ×80.

intensity and the di-glycosylated polypeptide is the most prominent of all three isoforms (Collinge *et al.*, 1996).

Discussion

CWD, like all other TSEs, is characterized by a long incubation period, which in deer is seldom less than 18 months (Williams and Young, 1992). In an experimental study of cattle inoculated intracerebrally with CWD from mule deer (first passage), amplification of PrP^{res} was demonstrated in only five of 13 (38%) cattle, after incubation periods that ranged from 23 to 63 months (Hamir *et al.*, 2001a, 2005a). In contrast, all inoculated cattle in the present study were positive for PrP^{res} within 16.5 months. This increased attack rate with shorter incubation periods probably indicates adaptation of the $CWD^{mule\ deer}$ agent to a new host. It could also be argued that the inoculum used for the primary passage (Hamir *et al.*, 2001a, 2005a) had a lower infectivity titre than that used for the second passage. However, the former successfully transmitted CWD to each of five white tailed deer within two years of intracerebral inoculation (Kunkle *et al.*, Unpublished).

In cervids, clinical CWD is characterized by emaciation, changes in behaviour, and excessive salivation (Williams and Young, 1992). Although the latter was not observed in the CWD inoculated cattle, all animals showed anorexia and considerable weight loss. Five cattle also showed intermittent neurological signs. Although none of these animals showed histopathological changes in the brain, all were shown to be positive for PrP^{res} by the IHC and WB methods. The presence of isolated vacuoles in the red nucleus is regarded as an incidental finding in cattle (McGill and Wells, 1993).

The uniform susceptibility, relatively short incubation, and absence of microscopical lesions in cattle given CWD brain material passaged once through cattle resembled findings in cattle inoculated intracerebrally with the scrapie agent (Cutlip *et al.*, 1997). In that experiment, 100% of cattle died 14–18 months after inoculation with material from the first cattle-passage of a US strain of the scrapie agent; none showed microscopical lesions and all were positive for PrP^{res}.

In the present experiment, the possibility that the PrP^{res} seen in tissue sections represented residual CWD material from the inoculum was ruled out because of the multifocal distribution of

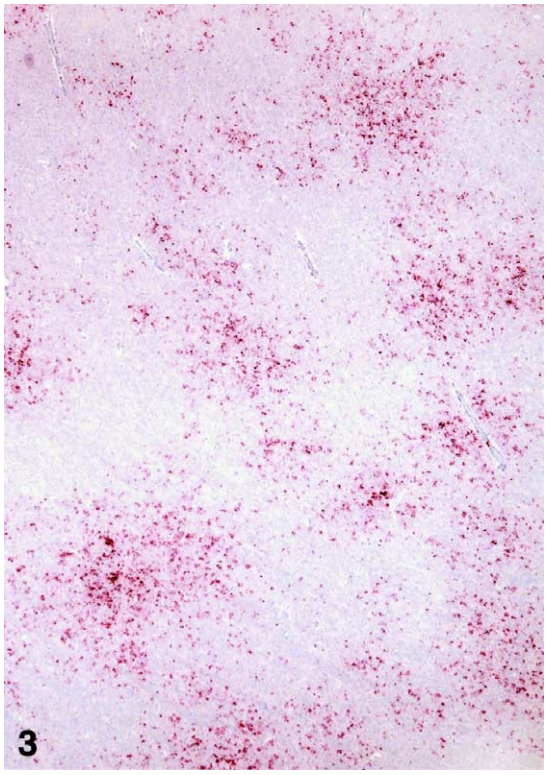

Fig. 3. Brain (midbrain) of animal 589. There are numerous foci of PrP^{res}. IHC labelling. ×80.

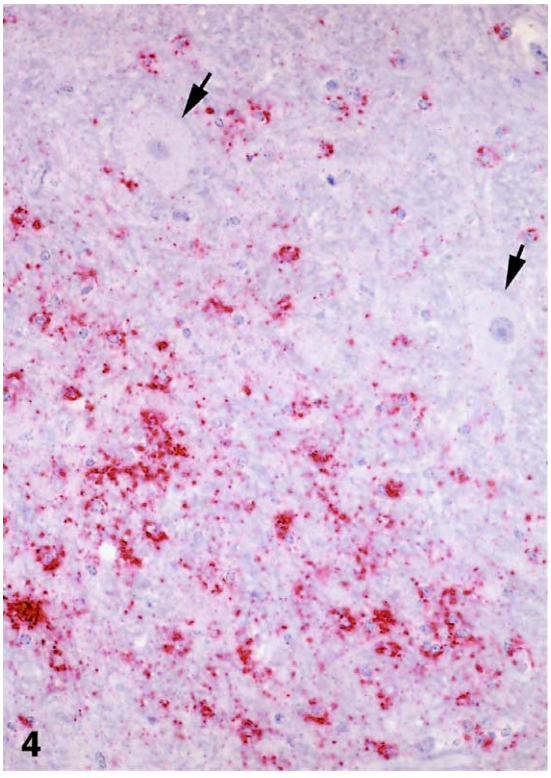

Fig. 4. Brain (midbrain) of animal 589. Higher magnification of one focus of labelled PrP^{res} from Fig. 3. Note the absence of PrP^{res} within neurons (arrows). IHC labelling. ×160.

PrP^{res} throughout the brain (excluding cerebellar folia) and cervical spinal cord of most of the affected animals. Had the PrP^{res} represented residual inoculum, it would probably have been confined to the sites of deposition in the midbrain or cerebrum. Moreover, in studies on sheep scrapie, Hamir *et al.* (2002) showed that intracerebrally inoculated brain material containing PrP^{res} was present for only a few days in sufficient quantity to be detectable immunohistochemically.

The present work confirms previous observations that PrP^{res} IHC labelling in cattle inoculated with the mule deer CWD agent is multifocal and glial cell-associated. This unusual pattern was first reported in descriptions of the primary CWD transmission to cattle (Hamir *et al.*, 2001a, 2005a), and the study described here showed that it was maintained through the second passage in cattle. Further studies now in progress will determine whether this feature also characterizes CWD transmission to cattle from other cervid species other than mule deer, namely, white tailed deer and elk.

In this and an earlier study of CWD in cattle (Hamir *et al.*, 2001a), IHC labelling differed from that seen in cattle with BSE or experimental transmissible mink encephalopathy (TME), both of which are associated with widespread diffuse labelling of grey matter neuropil, with labelled particles that are not obviously cell-associated except occasionally at neuronal cell membranes (Wells and Willsmith, 1995; Hamir *et al.*, 2005a). The IHC pattern in bovine CWD also contrasts markedly with that seen in scrapie-inoculated cattle, in which intracytoplasmic labelling of neurons is a prominent feature (Cutlip *et al.*, 1994, 1997).

When brainstems of CWD-infected cattle were analysed by WB for the presence of PrP^{res}, only three of six samples were found to be positive (Table 1). In contrast, all samples from the midbrain area were positive by this technique (Table 1; Fig. 5). It was noteworthy, however, that both brainstem and midbrain sections of all animals infected with CWD gave positive IHC results (Table 1) and a positive WB was associated with strong IHC labelling. This may indicate that the IHC procedure is more sensitive than the WB method for cattle-passaged CWD. However, given the multifocal nature of PrP^{res} distribution in the CNS of CWD-infected cattle, this result is not surprising. WB analysis requires a small sample of brain tissue (e.g. 0.2 g, as in the present study) to produce a 10% homogenate; approximately 10 µl (1 mg brain tissue equivalent) of this homogenate are loaded on to an SDS-PAGE gel for further

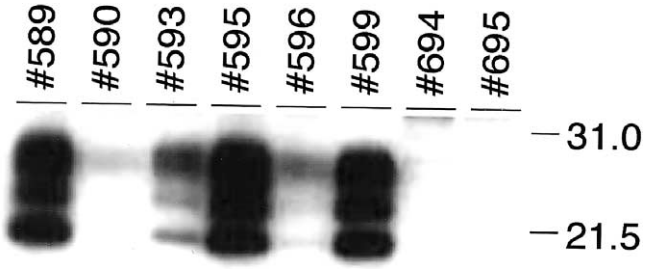

Fig. 5. Western blot analysis with monoclonal antibody 6H4 showing distinct profile of PrP^{res} in the five clearly positive animals (589, 593, 595, 596 and 599). Animal 590 is weakly positive. No specific signal is seen in animals 694 and 695, both non-inoculated control animals and classified as WB-negative. Molecular weight markers in kDa are indicated on the right side of the blot.

analysis. Bearing in mind the multifocal pattern of PrP^{res} distribution, the brain tissue used for the preparation of WB homogenate, unlike the large amount examined in the IHC procedure, might well contain few if any foci of PrP^{res} deposition, whereas the larger piece of tissue section used for IHC may contain detectable PrP^{res}. In this respect, therefore, the IHC method would seem preferable to the WB procedure and to other procedures (e.g. ELISA-based tests) in which only small amounts of tissue are used for analysis.

In comparison with experimental TME in cattle (Hamir *et al.*, 2005b), the experimental bovine CWD in this study was associated with less extensive IHC labelling in non-CNS (i.e. other than brain and spinal cord) neural tissues. Whereas the retina was positive in all cattle inoculated with TME, none of the CWD-infected cattle in this experiment had any retinal labelling. Similarly, in the present study there was no labelling in the pituitary gland, a tissue sometimes positive in TME-infected cattle. Because the incubation time for second passage CWD transmission (mean of 468 days) was only slightly longer than for TME (mean of 430 days), it seems likely that these different tissue affinities reflect a biological difference between these two TSE agents.

PrP^{res} IHC labelling was not observed in striated muscles (heart, tongue, masseter, diaphragm) of the experimental animals. This observation accorded with our previous findings (Hamir *et al.*, 2004a) in which striated muscle tissues from 20 animals (cattle, sheep, elk and raccoons) were examined for PrP^{res}. In these animals, all of which had developed a TSE after experimental inoculation, PrP^{res} was found by IHC examination in the brains, but not in muscle tissues. However, recent investigations with an enriched WB technique (Mulcahy *et al.*, 2004) have enabled us to detect PrP^{res} in the tongues of some sheep and elk experimentally inoculated with scrapie and CWD, respectively. This technique failed, however, to detect PrP^{res} in cattle inoculated with CWD or TME (Bessen *et al.*, unpublished). This study is still in progress, and the tongues of TSE-infected animals are currently being tested after careful removal from the carcasses to ensure non-contamination with infected brain material.

The present study and a previous experiment (Hamir *et al.*, 2005a) have established the biological characteristics of the $CWD^{mule\ deer}$ agent in cattle. However, isolates of CWD from other cervids (e.g. $CWD^{white\text{-}tailed}$ and CWD^{elk}) may differ. Transmission experiments with different CWD isolates are therefore needed to examine the possibility of variation in the CWD agent in wild cervids. Such experiments have recently been initiated at the National Animal Disease Center (NADC).

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